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# Identification and characterisation of canine ligament progenitor cells and their extracellular matrix niche

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Abstract

Ligaments are prone to injury and degeneration in humans and animals, however the healing potential of ligament is poor and current treatment options ineffective. Stem cell-based therapies hold potential for treatment of ligament injuries. This study aimed to characterise a ligament progenitor cell (LPC) population and to identify specific niche components which could promote the survival and function of LPCs. LPCs were isolated from canine cranial cruciate ligament and characterised for clonogenicity, multipotency and marker expression. The extracellular matrix (ECM) composition was characterised by the novel application of a metabolic labelling and mass spectrometry technique. LPCs demonstrated clonogenicity, multipotency and stem cell marker expression. A number of different collagens, glycoproteins and proteoglycans were identified in the LPC niche using proteomics. Metabolic labelling of cells demonstrated unique turnover profiles for distinct ECM protein groups, indicating the importance of certain niche components for LPC survival and function. The newly synthesised niche components identified in this study could be exploited to aid identification of LPCs and to promote their survival and function for potential ligament repair strategies.

Keywords

Ligament; matrix; proteomics; turnover; stem cells

## Introduction

Musculoskeletal soft tissues such as ligament are primarily composed of extracellular matrix (ECM) within which ligament cell populations reside. These tissues are prone to injury and degeneration, particularly the anterior cruciate ligament (ACL) [1], with an incidence of approximately 37 ACL ruptures per 100,000 people [2] and a greater incidence among athletes [3]. However, current treatment strategies for ACL repair are often ineffective and the healing potential of ACL is poor [4]. ACL injury can cause a loss of knee joint stability leading to considerable morbidity and ultimately osteoarthritis [5-7]. Ligament injury is also a common problem in comparative species such as dogs. Rupture of the cranial cruciate ligament (CCL), comparable to the human ACL, is the predominant cause of canine hind limb lameness [8]. Study of the canine CCL is also important for its translation into humans as a model for ACL disease [9-10]. Current treatment strategies for human ACL and canine CCL injuries have variable success rates [11-16], therefore a more effective therapeutic option for treatment of ACL and CCL injury is currently being sought.

The identification of a population of cells within ACL which possess stem cell properties [17] holds therapeutic potential for ligament repair in humans and dogs. Ligament-derived stem cells (LPCs) isolated from human ACL express stem cell and tenogenic markers [18-20], form colonies [21] and differentiate into osteogenic, adipogenic and chondrogenic cell types [17-18]. Human periodontal and rabbit medial collateral ligament LPCs have been used in tissue engineering strategies, and to treat human periodontal and rat medial collateral ligament injuries [22-24] with promising results, indicative of the potential of LPCs for treatment of ACL injuries.

The stem cell niche is the environment in which stem cells reside and consists of a number of different cellular and molecular factors [25]. One factor is the protein composition of the extracellular matrix (ECM) which has been shown to be integral for stem cell survival and function in a number of different cell populations. For example, the role of tenascin C in the neural stem cell niche [26-27], fibronectin in the haematopoietic stem cell niche [28-30] and fibromodulin in the tendon stem cell niche [31]. The role of ECM proteins in stem cell regulation in these tissues, particularly tendon, is suggestive of the importance of the LPC niche in LPC regulation. There are a number of techniques that can be utilised to investigate the stem cell niche, one of which is mass-spectrometry based proteomics [32-33]. Label-free mass spectrometry can be used for protein identification and quantification of ECM components in ligament and tendon [34-35]. Label-based mass spectrometry methods can be used to measure protein dynamics [36-37]. Proteomic analysis of a cell or tissue provides only a snapshot of that cell or tissue's proteome at a single point in time. The proteome is constantly changing as proteins are synthesised and degraded reflecting the developmental, physiological and pathological status of the cell or tissue [36, 38]. Therefore, the investigation of proteome dynamics is integral to fully understand the function and role of proteins within their niche.

This study aimed to characterise LPCs isolated from canine CCL for clonogenicity, multipotency and marker expression, and to identify components of the LPC niche with potential to promote LPC survival and function. This was achieved using label-free mass spectrometry for protein identification and quantification, and dynamic SILAC for investigation of the rates of protein synthesis and turnover in the niche. Characterisation of

LPCs from canine CCL and proteomic determination of the LPC niche has not previously been reported, neither has the use of dynamic SILAC to characterise the niche of any cell population. We hypothesised that LPCs isolated from canine CCL would demonstrate the hallmark properties of stem cells and would generate a specific ECM niche, but and that this niche would be dynamic in order to meet the changing cellular demands of stem cell populations.

## Experimental Procedures

### Isolation of canine ligament cells

CCLs were harvested from 9 disease-free canine cadaveric stifle joints (animals aged 3-7 years) which were euthanased for purposes not related to this study and were clinical waste material donated to the University of Liverpool. Ethical approval for use of this material in this project was granted by the local ethics committee (VREC159 and RETH00000553). The tissue was dissected into small pieces and digested overnight at 37°C in 1 mg/ml collagenase II. The resulting cell suspension was strained and the cells were resuspended in complete DMEM (DMEM supplemented with 10% foetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 µg/ml amphotericin B). For LPC isolation the cells were seeded at 1200 cells/$cm^2$ onto plates previously coated with 20 µg/ml human fibronectin and the media (complete DMEM + 5 ng/ml FGF-2) replaced after 20 minutes [39-40]. The cells were cultured at 37°C, 5% $CO_2$ and 5% $O_2$ for 10-12 days until they formed colonies. The colonies were isolated using 0.05% trypsin and transferred to T25 culture flasks [39]. All experiments were performed at passage 2-3.

**Colony formation assay**

Ligament-derived cells from 9 dogs were seeded at 100 cells/cm$^2$ in 6-well cell culture plates. After 7 days in culture the cells were fixed with 6% glutaraldehyde and stained with 0.5% crystal violet solution [41]. Colonies were imaged using a biomolecular imager (Typhoon FLA 7000; GE Healthcare, Illinois, USA), and analysed using ImageQuant software (GE Healthcare) for colony number and size.

**Tri-lineage differentiation assays**

Ligament-derived cell monolayers from 9 dogs were cultured for 21 days in osteogenic (complete DMEM containing 100 nM dexamethasone, 10 mM β-glycerophosphate and 50 mM ascorbic acid) [42] or adipogenic (complete DMEM containing 1 µM dexamethasone, 100 µM indomethacin, 10 µg/ml insulin and 500 µM IBMX) [17] induction media. Cell pellets (containing 5x10$^5$ cells) were cultured for 21 days in chondrogenic (complete DMEM containing 100 nM dexamethasone, 25 µg/ml ascorbic acid, 10 ng/ml TGF-β3 and ITS+3 supplement) [43] induction media. All control cells were cultured in complete DMEM for 21 days. Subsequently cells were stained with alizarin red to assess osteogenic differentiation and Oil Red O to assess adipogenic differentiation as described in the PromoCell MSC application notes (http://www.promocell.com/downloads/application-notes/). Chondrogenic pellets were paraffin embedded and 4 µm sections taken, which were rehydrated and stained with 1% Alcian blue solution. Cell pellets were also digested in 10 U/ml papain solution for 3 hours at 60°C before the total sulphated glycosaminoglycan (GAG) content was quantified. Dimethylmethylene dye was added to each sample and the absorbance read immediately at 570 nm. The GAG content was calculated from a standard

curve produced using chondroitin sulphate standards [44]. RNA was extracted from all assays to analyse lineage-specific gene expression.

**RNA extraction and qRT-PCR**

RNA was extracted from ligament-derived cells from 9 dogs using Trizol. cDNA was synthesised in a 25 µl reaction from 1-2 µg of total RNA. The conditions for cDNA synthesis were as follows: incubation at 5 minutes at 70°C, 60 minutes at 37°C and 5 minutes at 93°C with M-MLV reverse transcriptase and random-hexamer oligonucleotides [45-46]. qRT-PCR was conducted using a GoTaq(R) qPCR Master Mix, and in a 25 µl reaction 10 ng of cDNA was amplified in an AB 7300 Real Time PCR System (Applied Biosystems, California, USA). After an initial denaturation for 10 minutes at 95°C, 40 PCR cycles were performed consisting of 15 seconds at 95°C and 1 minute at 60°C [45-46]. Relative gene expression was calculated according to the comparative $C_t$ method [47]. Canine specific primers (Table 1) were designed using Primer-BLAST (NCBI) and the quality of each primer was tested using NetPrimer (Premier Biosoft). In addition, each primer was subjected to a BLAST (NCBI) search to ensure specificity. The best housekeeping gene was determined using the geNorm algorithm [48] and all primers were tested for efficiency; efficiencies between 90-110% were deemed to be acceptable.

Table 1. Primer sequences for canine genes.

| Gene | Forward | Reverse |
|---|---|---|
| GAPDH | CTGGGGCTCACTTGAAAGG | CAAACATGGGGGCATCAG |
| CD90 | TGTGCTCAGAGACAAACTGGT | CAGCCAGTCACAGGGAGATG |
| CD73 | ATGGCTCCACTCAATCCTGC | TCCCAGGTAATTGTGCCGTT |

| | | |
|---|---|---|
| CD105 | GACGCCGAGGTGACATACAT | GCTCTGACAGCTCCCTTGAG |
| CD44 | ACCTTCCAACTGCATACCCG | TCGTGGTCTTTGGTAATGGGG |
| SCX | GTCCAGCTACATCTCGCACC | GTCCAGCTACATCTCGCACC |
| MKX | GCGACCCCGGAGTTCTTC | CGCGGTCCTCAAAAAGCAC |
| OCT-4 | GAGGCTCTGCAGCTCAGTTT | AGCCCAGAGTGGTGACAGAC |
| TNMD | CCCACTCTAATAGCAGTTTCAGA | TCCTCACTTGCTTGTCTGGT |
| RUNX2 | GAACCCAGAAGGCACAGACA | ACTTGGTGCAGAGTTCAGGG |
| FABP4 | ATCAGTGTAAACGGGGATGTG | GACTTTTCTGTCATCCGCAGTA |
| COL2A1 | AGCTAAAGGATCTGCTGGCG | CTTGTTCGCCTTTGAAGCCA |

**$^{13}$C metabolic labelling of ECM proteins for proteomic analysis**

Ligament-derived cells isolated from 3 dogs were plated at 6x10$^4$ cells/cm$^2$ in complete DMEM (phenol red free) and incubated for 24 hours. Cells were then incubated in complete SILAC media (Sigma Aldrich, Missouri, USA) (SILAC media supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 2 µg/ml amphotericin B, 0.2 mM ascorbate and 0.4 mM β-aminopropionitrile) as well as 0.8 mM [$^{12}$C]L-lysine 2HCl, 0.4 mM [$^{12}$C]L-arginine HCl and 0.4 mM [$^{12}$C]L-proline (ThermoFisher Scientific, Massachusetts, USA)) (unlabelled media) for 1 hour. The media was then exchanged for complete SILAC media supplemented with 0.8 mM [$^{13}$C]L-lysine 2HCl, 0.4 mM (ThermoFisher Scientific) [$^{12}$C]L-arginine HCl and 0.4 mM [$^{12}$C]L-proline (labelled media) for 4, 24 and 48 hours. After which the cells were removed using trypsin and the plates washed with PBS.

**Extracellular matrix extraction**

Rapigest (Waters, Massachusetts, USA) solution (0.06% (w/v) solution in 25 mM $NH_4HCO_3$) was applied to each plate and incubated for 30 minutes at room temperature before incubation at 80°C for 10 minutes [49]. Protein extracts were reduced by the addition of 10 µl of 60 mM DTT in 25 mM $NH_4HCO_3$ followed by sample incubation at 60°C for 10 minutes. Alkylation was carried out by the addition of 10 µl of 180 mM iodoacetamide in 25 mM $NH_4HCO_3$ and the sample incubated at room temperature for 30 minutes in the dark. 10 µl of 0.05 µg/µl trypsin was added to samples before incubation at 37°C overnight. Digests were terminated by the addition of trifluoroacetic acid and incubated at 37°C for 45 minutes, before centrifugation at 17,200 g for 30 minutes and transfer of the clarified digest to fresh low-bind tubes.

**Liquid chromatography- tandem mass spectrometry (LC-MS/MS)**

Samples (ECM extracts from cultured cells) were randomised and run on a 1 hour gradient with a 30 minute blank between samples to eliminate contamination. For instrument performance evaluation, E.coli digest standards spiked with RePLiCal [50] were included before and after the run. Data-dependent LC-MS/MS analyses were conducted on a QExactive quadrupole-Orbitrap mass spectrometer (ThermoFisher Scientific) [51-52] coupled to a Dionex Ultimate 3000 RSLC nano-liquid chromatograph (ThermoFisher Scientific). Further detail is given in Supporting Information.

**Proteomic data analysis**

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [53] partner repository

(http://www.ebi.ac.uk/pride/archive/) with the dataset identifier PXD008602 and 10.6019/PXD008602.

For protein identification and label-free quantification raw data files were imported into PEAKS v.8 (Bioinformatics Solutions Inc, Waterloo, Canada) [54] and *de novo* and database PEAKS searches were carried out using the UniProtKB canine protein database (EMBL-EBI, Hinxton, UK) [55]. Protein identifications and gene ontology were analysed using the UniProtKB canine protein database and the Matrisome Project database v.2 (Massachusetts Institute of Technology, Cambridge, Massachusetts) [56] and protein interaction network analysis was performed using STRING software v.10.5 (STRING Consortium 2017).

For analysis of heavy isotope metabolic labelling, peptides for ECM proteins labelled with $^{13}$C lysine were initially identified using MASCOT v.2.6 (Matrix Science, London, UK). Raw data files were imported into Xcalibur v.3.0.63 (ThermoFisher Scientific) for analysis of extracted ion chromatograms and raw mass spectra. Heavy (H) and light (L) peaks were identified for each labelled peptide based on the observed m/z ratio and the scan number obtained from MASCOT. The area under each peak was recorded and relative isotope abundance (RIA) (the proportion of the total protein pool labelled with heavy isotope) was calculated as H/(L+H), indicating the level of new protein synthesis. The total protein pool was calculated as H+L, indicating the level of protein turnover. Further detail is given in Supporting Information.

**Statistical analysis**

Statistical analysis of stem cell characterisation data was performed using SigmaPlot (Systat Software Inc, California, USA)). Shapiro Wilk tests for normality were performed. For normally distributed data parametric tests were used for pairwise comparisons. For data

which was not normally distributed $Log_{10}$ data transformations were performed before normality was confirmed and parametric tests used. For comparing two groups paired or independent Student's t-tests were used, as appropriate.

Statistical analysis of label-free protein quantification data was performed by PEAKS using an ANOVA. Proteins were considered to be significantly different between groups using a -10logP score of 15, a fold change of ≥ 2 and a quality score ≥ 0.2. For statistical analysis of metabolic labelling data a two-way ANOVA with repeated measures and a Holm-Sidak post-hoc test was used. p-values of <0.05 were taken to be statistically significant for all data.

## Results

### LPCs display clonogenicity and stem cell marker expression

Ligament-derived progenitor cells grew in heterogeneous dense colonies (Fig.1.A-C.) and showed a rounded and fibroblastic morphology upon initial plating (Fig.1.D.), with the rounded morphology lost with passaging. The gene expression of stem cell (OCT4, CD105, CD44, CD90 and CD73) and tenogenic (SCX, MKX and tenomodulin) markers was assessed by qRT-PCR (Fig.1.E.). Expression of Oct4 in LPCs was low, however CD marker expression was much higher. Tenogenic markers were expressed at much lower levels than CD markers. The expression of the haematopoietic markers CD34 and CD45 was also generally low (Fig.1.E.).

### LPCs display dual-lineage differentiation potential

LPCs demonstrated signs of osteogenic, adipogenic and chondrogenic differentiation as assessed by alizarin red, oil red O and alcian blue staining respectively (Fig.2.A.). Gene expression analysis of lineage specific genes showed a significant increase, between control

and induced cultures, in the osteogenic marker RUNX2 ($p=0.004$), the adipogenic marker FABP4 ($p=<0.0001$) as well as an increase in the chondrogenic marker COL2, however this was not significant (Fig.2.B.). There was an increase in GAG content between control and induced samples from 1.6 (±1.0) µg to 2.1 (±1.9) µg, however this increase was not significant (Fig.2.C.).

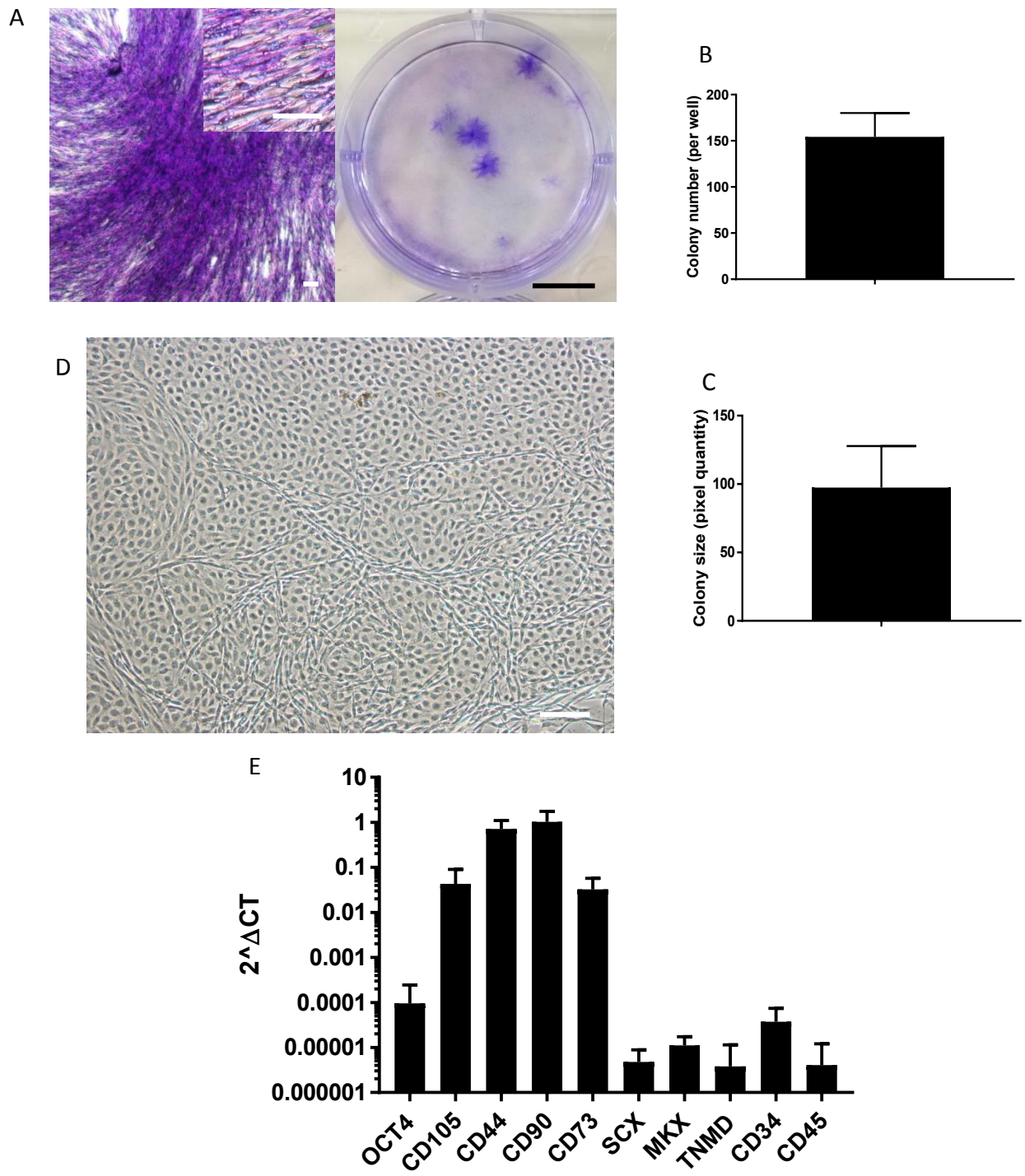


Figure 1. Canine LPC morphology, clonogenicity and stem cell marker expression. Representative images of cell morphology and colony formation are shown. White bars = 100 µm, black bars = 1 cm (A). Colonies were counted (B) and measured (C) using ImageQuantTL software. Error bars shown represent SD. Representative images shown of LPCs after initial plating to emphasise differences in morphology. White bars = 100 µm (D). Gene expression analysis of stem cell markers in LPCs was performed using qRT-PCR (E). Values are shown on a logarithmic scale and normalised to GAPDH. n=9 biological replicates.

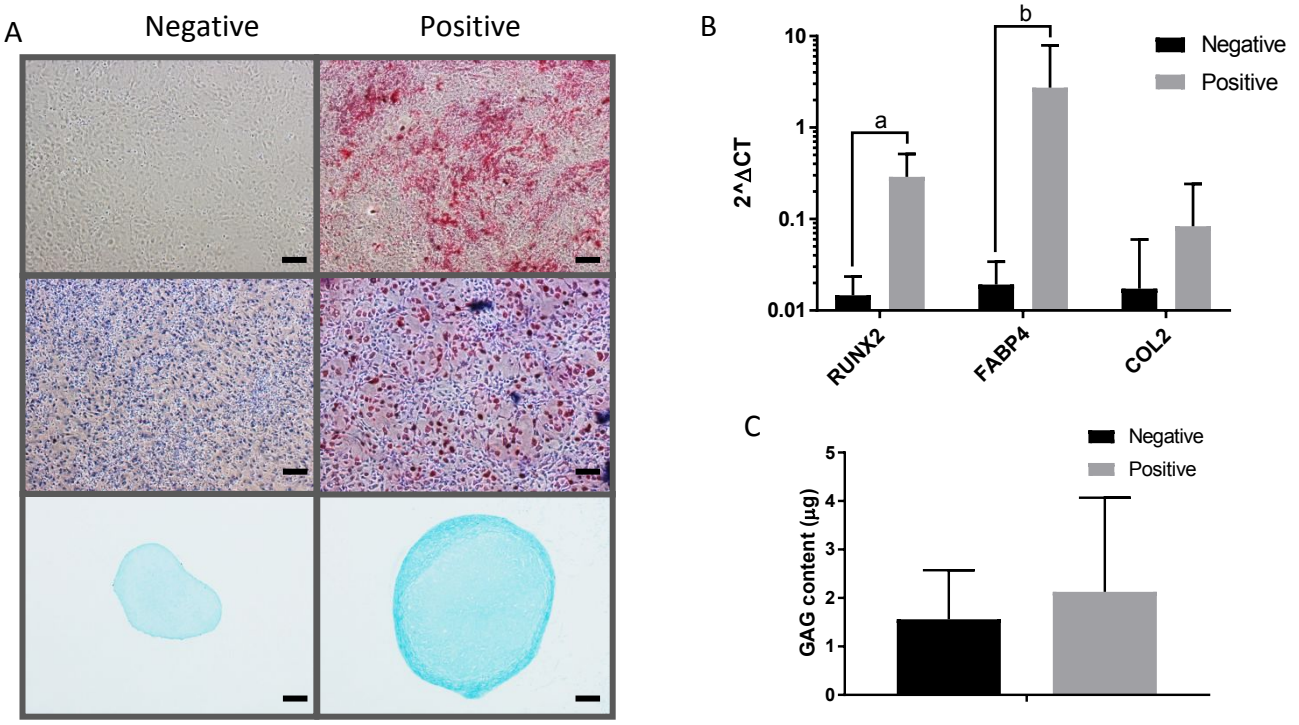


Figure 2. Dual-lineage differentiation potential of canine LPCs. Representative images are shown for histological staining of cells after induction of osteogenic, adipogenic and chondrogenic differentiation (positive) and for control samples (negative) (A). Cells subjected to osteogenic media were stained for calcium deposits using alizarin red (AR). Cells subjected to adipogenic media were stained for oil droplet formation using oil red O (ORO), and cell pellets exposed to chondrogenic media, for GAG formation using alcian blue (AB). Bar = 100 µm. Gene expression analysis of lineage specific markers was performed using qRT-PCR (B). Values are shown on a logarithmic scale and normalised to GAPDH. Error bars shown represent SD. $^{a}p = 0.001$, $^{b}p = 0.001$. Total GAG content of cell pellets with (positive) or without (negative) chondrogenic induction was measured (E). n=9 biological replicates.

**A variety of ECM proteins were identified in the LPC niche**

A total of 75 ECM related proteins were identified by PEAKS software and the Matrisome Project database as being produced by LPCs. The list of proteins is provided in Table S1. and the proportion of proteins belonging to each ECM category is shown in Figure 3. The Matrisome Project categorised proteins into ECM collagens (10 proteins), ECM proteoglycans (6 proteins), ECM glycoproteins (14 proteins), ECM affiliated proteins (14 proteins), ECM regulators (25 proteins) and secreted factors (6 proteins). A STRING protein interaction map showed strong interactions between collagens and some glycoproteins with weaker clusters forming for some regulatory and affiliated proteins (Fig.4).

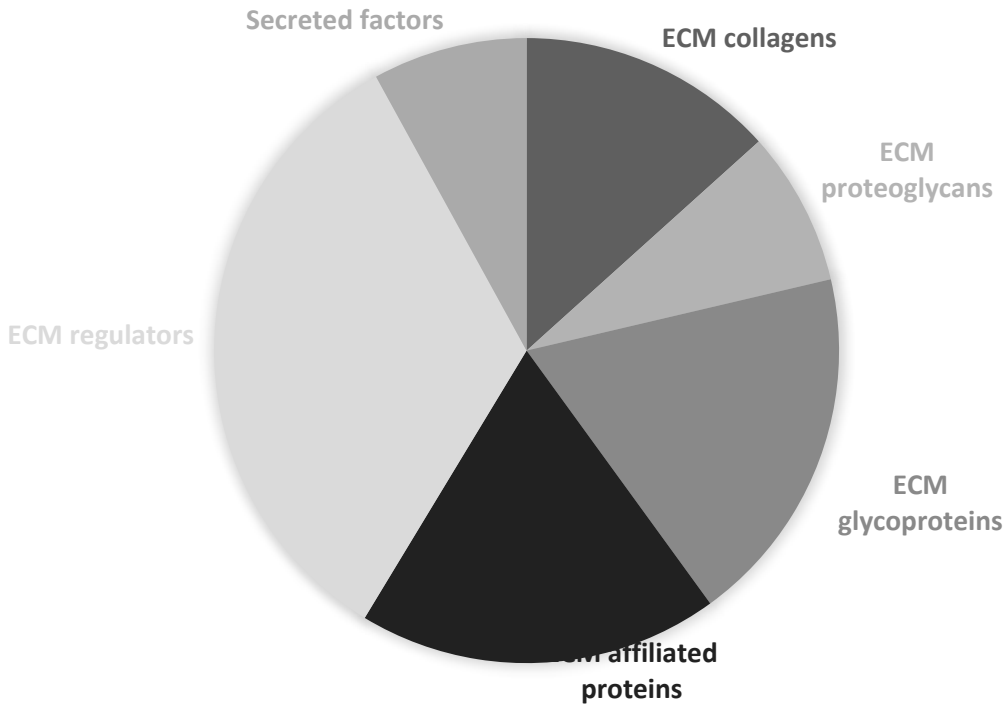


Figure 3. Identification of ECM proteins produced by canine LPCs. ECM proteins produced by LPCs, as determined by PEAKS software, were divided into matrisomal protein categories according to the Matrisome Project database.

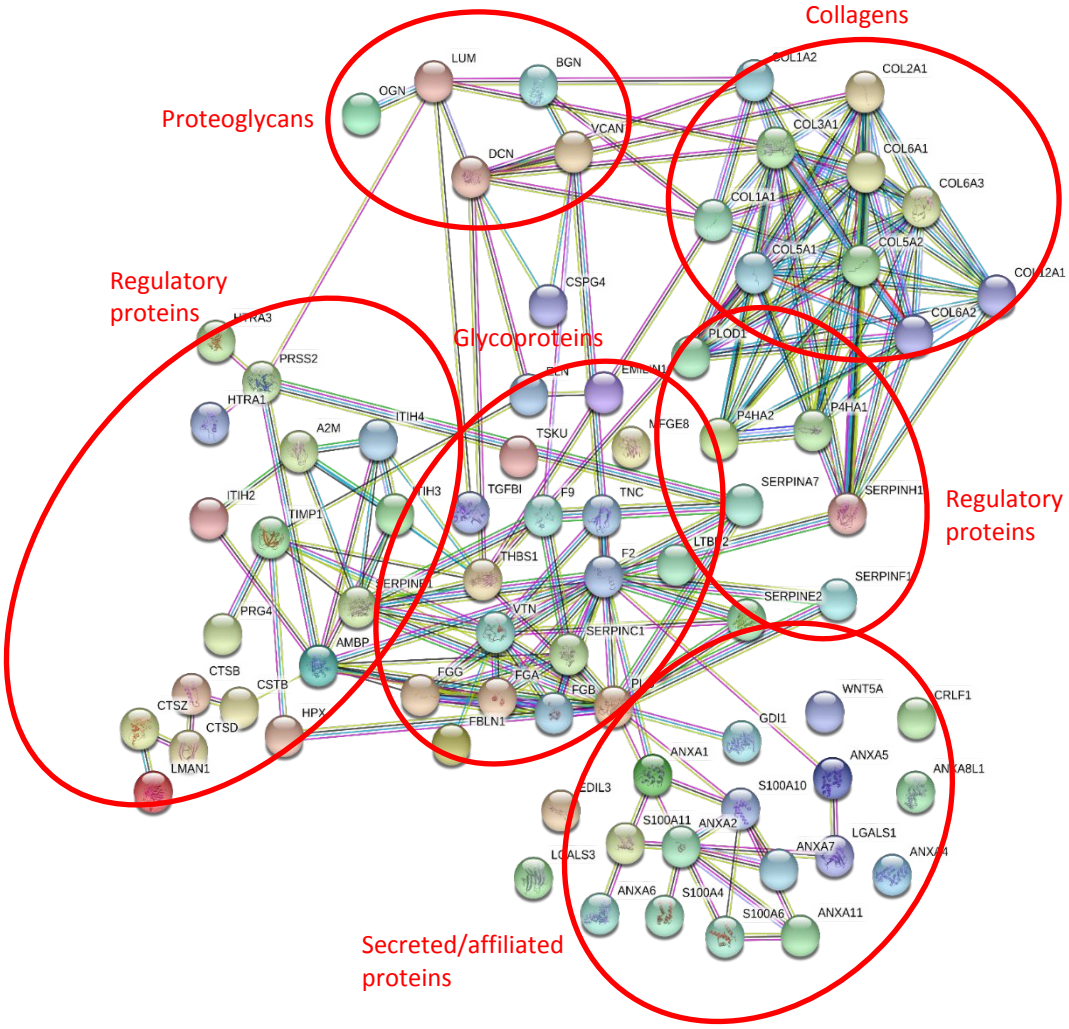


Figure 4. Canine ligament ECM protein interactions. All ECM proteins produced by LPCs as determined by PEAKS software and the Matrisome Project were entered into STRING for visualisation of ECM protein interactions.

**Metabolic labelling identified differences in dynamics between different ECM protein groups in LPCs**

ECM samples extracted from LPCs were analysed for new protein synthesis (heavy peptide content) and turnover (total peptide content) using LC-MS/MS after heavy isotope metabolic labelling. The data is presented as graphs demonstrating both total peptide quantity (total protein = heavy peptide + light peptide) and relative isotope abundance (RIA) which is the proportion of the total protein pool labelled with heavy isotope (calculated as heavy peptide/(light peptide + heavy peptide)) (Fig.5,6,7,S1).

There was new synthesis of collagen type I alpha 1 and 2, collagen type III alpha 1 and collagen type VI alpha 1, 2 and 3. The general trend for new protein synthesis consisted of an initial increase in collagen synthesis over the first 24 hours, with a decrease seen at 48 hours. This is consistent with the protein turnover data which suggested an increase in total protein quantity over the first 24 hours and a decline at 48 hours (Fig.5).

For proteoglycans new synthesis of decorin, lumican and tsukushi was observed and this generally increased over 48 hours. Consistent with this observation, total protein quantity also continued to increase over 48 hours (Fig.6).

For glycoproteins there was new synthesis of transforming growth factor β1 (TGFβ1), tenascin C, fibronectin and elastin. Synthesis of new glycoproteins generally continued to increase over 48 hours. The total protein pool varied between glycoproteins, with some proteins demonstrating an increase in total protein quantity over time (such as tenascin C), consistent with the synthesis of new protein. In contrast, some proteins showed an initial increase in total protein quantity over 24 hours followed by a decrease at 48 hours (such as fibronectin and elastin), indicative of increased protein degradation between 24 and 48 hours (Fig.7).

For ECM regulators new synthesis of HtrA serine peptidases 1 and 3, cathepsins B and D and serpin peptidase inhibitors E2 and H1 was observed. Synthesis of new regulatory proteins generally continued to increase over 48 hours. The total protein content for ECM regulatory proteins increased over 24 hours and then started to decrease by 48 hours, indicative of increased protein degradation between 24 and 48 hours (Fig.S1).

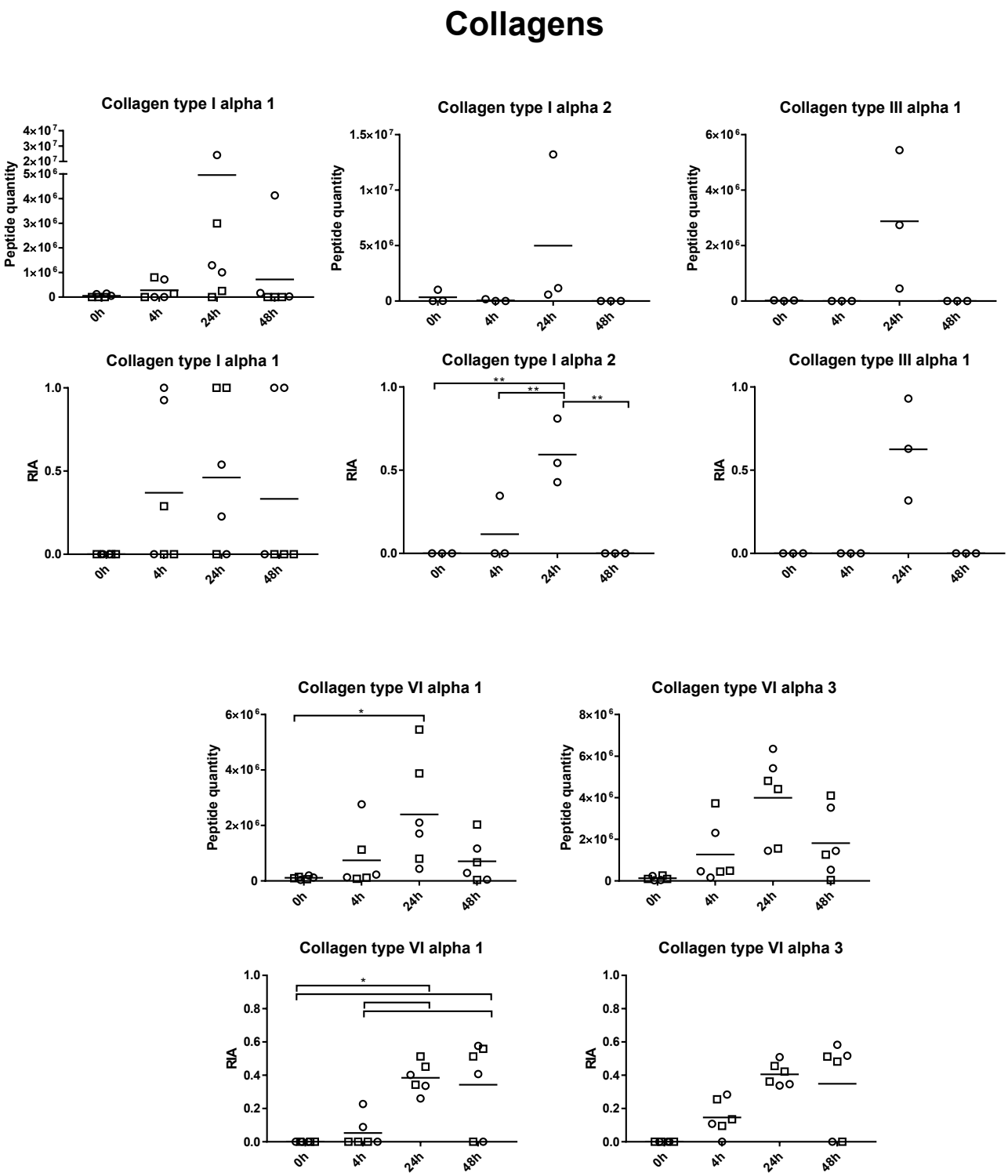


Figure 5. Metabolic labelling of canine LPC collagens. Cells were labelled with $^{13}$C lysine for 4, 24 and 48 hours before the ECM was harvested and LC-MS/MS performed. ECM proteins labelled with $^{13}$C lysine were identified using MASCOT and extracted ion chromatograms were analysed using Xcalibur. Heavy (H) and light (L) peaks were identified for each labelled peptide and the area under the peak recorded. Total peptide quantity (H+L) and relative isotope abundance (RIA) (H/(L+H)) are shown. Squares and circles represent different peptides. * = p<0.05, ** = p<0.01 and *** = p<0.001.

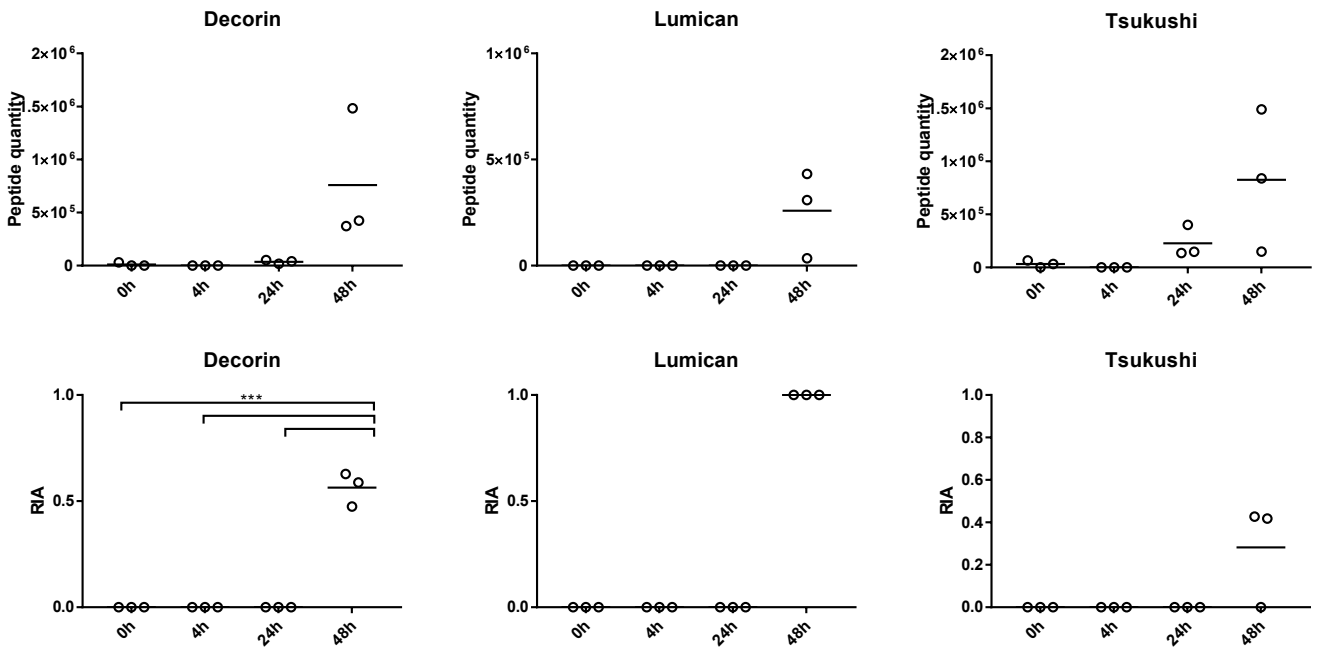


Figure 6. Metabolic labelling of canine LPC proteoglycans. Cells were labelled with $^{13}$C lysine for 4, 24 and 48 hours before the ECM was harvested and LC-MS/MS performed. ECM proteins labelled with $^{13}$C lysine were identified using MASCOT and extracted ion chromatograms were analysed using Xcalibur. Heavy (H) and light (L) peaks were identified for each labelled peptide and the area under the peak recorded. Total peptide quantity (H+L) and relative isotope abundance (RIA) (H/(L+H)) are shown. Squares and circles represent different peptides. * = $p<0.05$, ** = $p<0.01$ and *** = $p<0.001$.

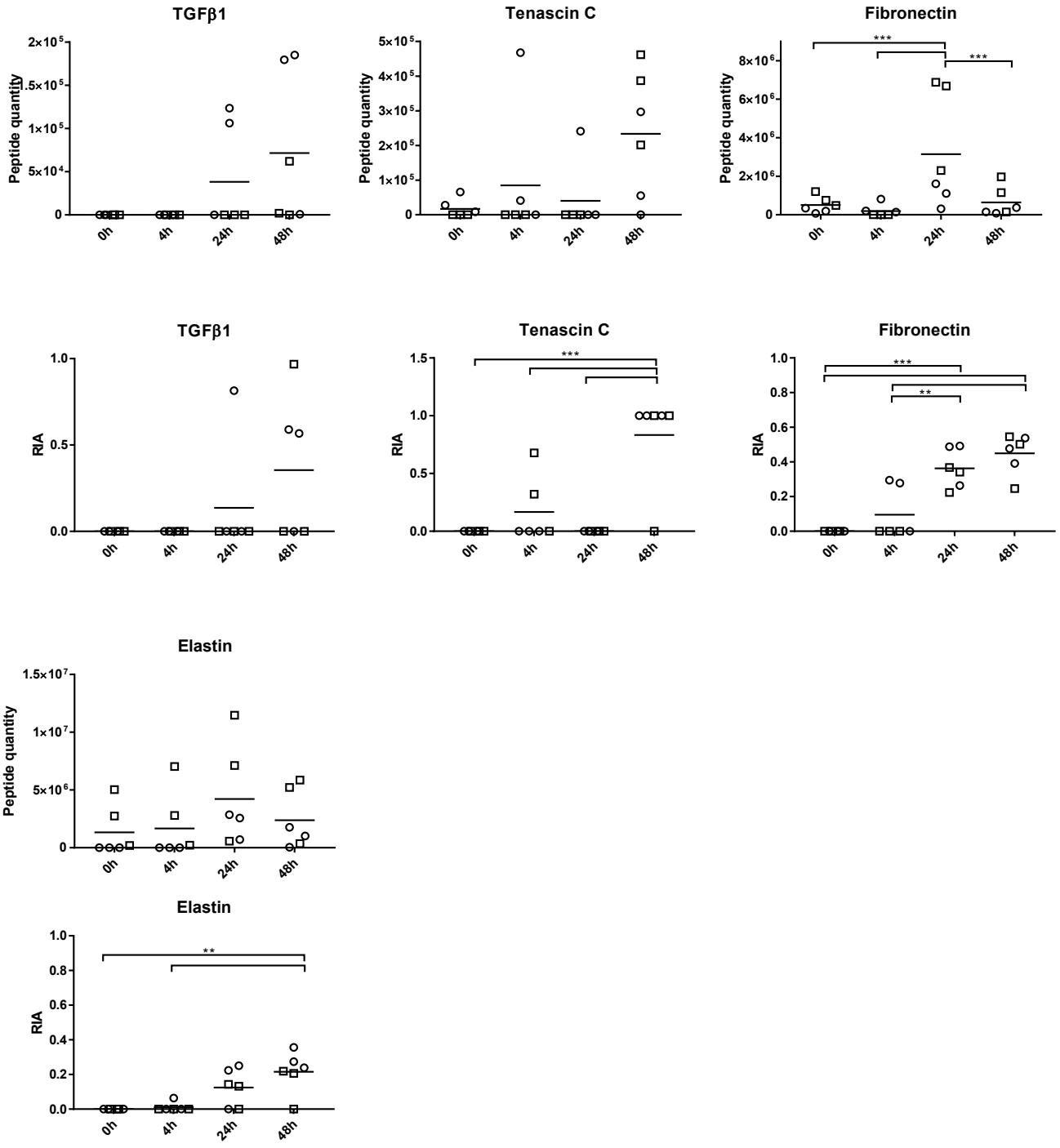


Figure 7. Metabolic labelling of canine LPC glycoproteins. Cells were labelled with $^{13}$C lysine for 4, 24 and 48 hours before the ECM was harvested and LC-MS/MS performed. ECM proteins labelled with $^{13}$C lysine were identified using MASCOT and extracted ion chromatograms were analysed using Xcalibur. Heavy (H) and light (L) peaks were identified for each labelled peptide and the area under the peak recorded. Total peptide quantity (H+L) and relative isotope abundance (RIA) (H/(L+H)) are shown. Squares and circles represent different peptides. * = p<0.05, ** = p<0.01 and *** = p<0.001.

## Discussion

We have successfully isolated a progenitor cell population from the canine cranial cruciate ligament (CCL) that demonstrates many of the defined properties of stem cells. This cell population can form colonies, self-renew, express stem cell markers and can differentiate into osteogenic and adipogenic cell types; all traditional properties of stem cells (Fig.1-2). To date no other studies have isolated such cells from canine CCL and the identification of such a cell population may hold potential for stem cell therapy. Our findings are consistent with previous studies in humans, with LPCs isolated from human ACL demonstrating the same properties as the cells isolated in this study [18-20]. Despite the stem cell properties demonstrated by the cell population selectively isolated in this study, it is likely that this is a heterogeneous population of cells, as clonal cell populations were not derived and different cell morphologies were observed upon initial plating.

The stem cell population we have isolated could however hold therapeutic potential for treatment of ligament injuries. Previous studies have investigated the use of periodontal and medial collateral LPCs for repair of ligament with successful outcomes [22-24, 57], however no studies have yet investigated the use of LPCs from ACL or CCL to repair cruciate ligament injuries. LPCs may provide therapeutic benefit through differentiation into fibroblasts and ECM production or through immunomodulatory effects on the damaged tissue as demonstrated in mesenchymal stem cells [58-59]. Identification of niche components which promote stem cell survival and function could provide potential therapeutic benefit by boosting the resident stem cells if introduced non-invasively to the damaged tissue *in vivo*.

The present study has, for the first time, characterised the canine LPC niche, demonstrating the presence of collagens, proteoglycans, glycoproteins and ECM related

proteins (Fig.3-4). Although the growth of cells in 2D cell culture on fibronectin substrates may not mimic the natural environment of ligament cells, the cells grown on these conditions displayed stem cell characteristics, therefore, the niche produced by these cells is supportive of a stem/progenitor cell phenotype. This indicates that the described approach is an informative model, which is further confirmed by the consistency of our findings with previous literature looking at the proteome of whole canine CCL [35] and human ACL [60] tissue. Various collagens (types I, III, V, VI, XII), decorin, biglycan, lumican, osteoglycin, fibrinogen, fibromodulin, tenascin C, thrombospondin 1, TGFB1, vitronectin and elastin were identified in these studies [35, 60], consistent with our findings. No studies to date have investigated the ECM proteome of intra-articular ligament cells in 2D culture. However, several studies have demonstrated gene expression of ECM components by cultured human ACL and periodontal LPCs [20, 61-62], consistent with this study. One limitation of this study was the lack of analysis of ECM proteins secreted into the media. The role of some of the ECM proteins identified in this study in other stem cell niches has previously been demonstrated. For example, the role of tenascin C in the neural stem cell niche [26-27], fibronectin in the haematopoietic and embryonic stem cell niche [28-30, 63] and fibromodulin and biglycan in the tendon stem cell niche [31].

The novel application of a metabolic labelling technique in this study enabled investigation of protein synthesis and turnover by LPCs (Fig.5-7). New protein synthesis was analysed by measuring the incorporation of heavy isotope into newly synthesised ECM proteins. This technique also provided information on protein turnover through analysis of total protein content. Synthesis and turnover of a number of ECM protein targets was observed, including collagens, proteoglycans, glycoproteins and ECM regulatory proteins. A general trend for collagens of an initial increase in new and total protein synthesis up to 24 hours followed by a decrease between 24 and 48 hours was observed. At 24 hours a variable

RIA was observed for different collagens with as much as 93% of collagen type I alpha 1 being turned over in a 24 hour period and 46% of collagen type VI alpha 3. Previous studies have reported turnover rates of: 0.045%/h and 0.04%/h in human patellar tendon and ACL using metabolic labelling [64]; a collagen half-life of 34-198 years in equine superficial digital flexor tendon by measuring aspartic racemization and collagen degradation [65]; and a lack of turnover in human Achilles tendon demonstrated using the $^{14}C$ bomb-pulse method [66]. These turnover levels are considerably lower than our observations. This discrepancy may be due to the use of tissue in previous studies and the use of *in vitro* cultured cells in this study. Alternatively, newly-synthesised procollagen may be secreted from the cells but not incorporated into longer-lived collagen fibrils. The decline in total and labelled peptide seen at 48 hours is due to both reduced synthesis and increased degradation. The reduction in collagen synthesis may be due to changes in specific regulatory enzymes, such as the collagen chaperone Hsp47 (Serpin H1, Fig S1) and the increase in collagen degradation is most likely due to upregulation of collagen degrading enzymes, however only minor collagen degradation enzymes were identified. For proteoglycans there were a limited number of peptides which had been labelled with heavy isotope, therefore the conclusions we can draw for these proteins are with less confidence. The trend for proteoglycan turnover generally consisted of a continual increase in both total and heavy labelled peptide content. Previous reports have demonstrated high turnover of proteoglycans in tendon and ligament [67-71], consistent with this study. In our study, synthesis of new glycoproteins followed a similar pattern to proteoglycans with a continual increase over time, however total protein content was variable. This variability could reflect differences in regulation of these proteins and differences in function. Previous studies in equine tendon demonstrated a slow rate of turnover for non-collagenous proteins with a half-life of 2-3.5 years in tissue [65]. In the present

study, synthesis of ECM regulatory proteins HtrA serine peptidase 1 and 2, cathepsin B and D and serpins E2 and H1 continually increased over time, whereas total protein content increased until 24 hours and then decreased between 24 and 48 hours. Little is known about the turnover of these ECM regulatory proteins, however due to their regulatory nature and the requirement for fast responsiveness to changing cellular demands it would be expected that their turnover would be high [72], as seen in this study. There are complex interactions between ECM core and regulatory proteins which may be reflected in their expression and turnover profiles. The high rate of turnover of ECM proteins seen in this study, when compared with other studies in tendon and ligament, is likely due to the intentional enrichment of stem/progenitor cells in culture and the requirement for faster proliferation and differentiation of stem/progenitor cells *per se*, with increased turnover of niche components providing an advantage [73].

A limitation of this study is the lack of validated canine antibodies available for both stem cell markers and ECM proteins. Therefore, the gene expression of stem cell markers has been analysed rather than the phenotype. In addition, the lack of cross-reactivity of antibodies for canine proteins means we are unable to validate our mass spectrometry protein identifications. However, for the metabolic labelling data each peptide detected by MASCOT was manually verified in Xcalibur in order to calculate RIA therefore we have confidence in the proteins identified by MASCOT. Although Western blotting would have been useful to confirm protein identifications, this method is not appropriate for detecting alternations in protein dynamics and therefore would not have been a suitable method of validation for this novel aspect of the study.

## Conclusions

In conclusion, we have, for the first time, utilised a proteomics-based method to analyse the dynamics of newly synthesised ECM proteins in the extracellular niche of cultured cells. The study of protein dynamics is of vital importance, for comprehensive protein identification and to understand the function and regulation of proteins. It also enables a comparison of the dynamics of different proteins and highlights potential protein interactions and roles based on protein synthesis and degradation profiles.

The identification of a stem cell population and investigation of the stem cell niche in canine CCL has not previously been reported. The novel cell population identified in this study may hold therapeutic potential for treatment of canine ligament injuries and the identified niche protein targets could be translated to therapies for human ligament damage. Future work should include further investigation of these novel protein targets and their role in the LPC niche as well as comparing the profile of stem cells and their niche in healthy and diseased ligament tissue.

## Authors' contributions

KJL acquired, analysed and interpreted data. DMS completed proteomic sample preparation and mass spectrometry and assisted with proteomic data interpretation. EJC, PDC and EGC-L designed the study. KJL drafted the paper. All authors critically revised the manuscript and read and approved the final submitted version.

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The authors declare no competing financial interest.

Supporting Information

The following supporting information is available free of charge at ACS website http://pubs.acs.org:

Table S1. Identification of ECM proteins produced by canine LPCs

Table S2. List of peptides for identified ECM proteins in LPCs

Figure S1. Metabolic labelling of canine LPC ECM regulators

Supporting experimental procedures: Liquid chromatography- tandem mass spectrometry;

Proteomic data analysis

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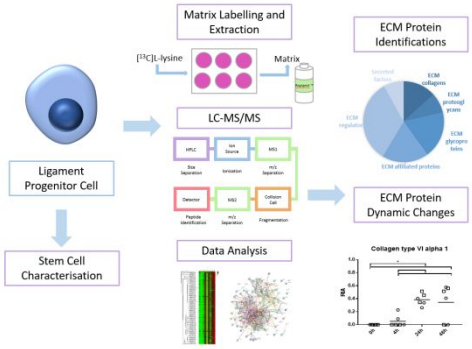
Matrix Labelling and Extraction
[13C]L-lysine
Matrix
LC-MS/MS
Ligament Progenitor Cell
Stem Cell Characterisation
Data Analysis
ECM Protein Identifications
ECM Protein Dynamic Changes
Collagen type VI alpha 1

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